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4 Title of the invention

Screening of Neisserial Vaccine Candidates Against Pathogenic Neisseria

5. Name of your agent *(if you have one)*

Swindell & Pearson

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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SCREENING OF NEISSERIAL VACCINE CANDIDATES AGAINST PATHOGENIC NEISSERIA

The present invention relates to vaccine candidates in Pathogenic *Neisseria*, and particularly but not exclusively to a screening system for the identification of $CD4^+$ T-cell stimulating vaccine candidates in Pathogenic *Neisseria*.

The term "vaccine candidates" is used to refer to peptides which may prove, upon further study, to exhibit some form of vaccine property. In particular, the vaccine candidates discussed below are peptides which stimulate $CD4^+$ T-cells (T-cells with CD4 marker on them).

The generic name Pathogenic *Neisseria* covers the pathogenic organisms *Neisseria meningitidis* and *Neisseria gonorrhoea*.

Neisseria meningitidis (the meningococcus) causes meningitis and overwhelming septicaemia that can kill within hours. It also causes outbreaks of meningococcal disease. *Neisseria gonorrhoea* (the gonococcus) causes gonorrhoea and other invasive diseases, e.g. pelvic inflammatory diseases and septic arthritis.

Although the two neisserial species (N. meningitidis and N. gonorrhoea) have evolved to colonise and invade different anatomical sites of the human body, they are strongly related and share extensive amount of genetic, immunochemical and other biological properties. They are believed to have evolved from a common ancestor, a view strongly supported by the recently released respective genomic sequence data. The outer membrane structure of the two organisms are very similar with a vast number of outer membrane proteins, including some vaccine candidates, being virtually identical. Recent data suggest that vaccines based on conserved (cross-reactive) immunogenic proteins may protect against both organisms.

The mechanisms responsible for the development of natural immunity to meningococcal disease remain unclear and the currently available capsular polysaccharide (CPS)-based vaccines provide only serogroup-specific and short-lived protection and are not effective in children under two years of age. Additionally, the CPS of serogroup B meningococci, which are responsible for the majority of cases in Europe and America, is only very poorly immunogenic in humans, generating mainly IgM antibodies.

Recovery from meningococcal infection is followed by long lasting immunity and, in the absence of immunodeficiencies, second episodes of meningitis (with homologous or heterologous strains) are extremely rare. This fact indicates that there are non-capsular (cross-reactive) antigens that can stimulate T-cell memory and thus generate a long-lasting and cross-protective immunity.

To achieve an efficient humoral immune response resulting in the production of high affinity IgG antibodies and the generation of memory B lymphocytes (B-cells), help from T lymphocytes (T-cells) is required. However, helper T-cells respond to peptide antigens associated with class II molecules of the major histocompatibility complex (MHC - designated HLA in humans) on the surface of antigen presenting cells. Therefore, they will not be stimulated by purified polysaccharide vaccines (T-cell independent B-cell immunogens). To trigger a strong memory T-cell response when the host confronts the virulent organism, the target B-cell epitope should be expressed along with helper T-cell stimulating epitopes. Identification and characterisation of the peptide epitopes that can best stimulate meningococcal specific $CD4^+$ T-cells is considered among the priorities that have long been ignored. An ideal meningococcal vaccine must consist of a carefully selected mixture of well-characterised B- and T-cell antigens capable of generating a long lasting immunity.

It appears that meningococcal vaccine candidates will also have the potential to protect against gonococcal disease.

In the following description the term T-cell clones is defined as the population of cells which originate from a single T cell.

In a first aspect, the present invention provides a method of generating T-cell lines and clones specific to neisserial proteins, the method comprising isolating peripheral blood lymphocytes (PBLs) from the peripheral blood of normal donors and patients recovering from neisserial disease, culturing the PBLs with neisserial proteins with or without a proliferation stimulant for a prescribed period, stimulating proliferation of T-cell lines and clones which are specific to neisserial proteins, and maintaining same by regular stimulation.

The neisserial proteins are preferably prepared from *Neisseria meningitidis* and/or *Neisseria gonorrhoea* grown under iron restrictions to induce the expression of iron-regulated proteins.

The peripheral blood is preferably obtained from naturally infected patients at different stages of illness. Preferably the stages include an acute stage (on admission), early convalescence (seven days after admission), late convalescence (six weeks after discharge) and after full recovery (3 months and twelve months after discharge).

Preferably the peripheral blood is heparinised and the PBLs may be isolated therefrom by centrifugation.

Preferably the PBLs are initially cultured in medium containing human serum. Preferably the PBLS are cultured with the neisserial proteins and Interleukin 2 (IL-2) for the prescribed period. Preferably the prescribed period is 3-10 days and may be 5 days.

Preferably IL-2 stimulates the proliferation of the activated T-cell lines and clones. Preferably the T-cell lines and clones are maintained by weekly stimulation. The stimulation may be provided by proteins in the presence of

IL-2 and feeder cells. Preferably the feeder cells are antigen presenting feeder cells and may be autologous Epstein-Barr virus transformed B-lymphocytes (EBVB).

The specificity of the T-cell lines and clones to neisserial proteins is preferably tested prior to storing in liquid nitrogen. Preferably the specificity is tested by measurement of tritiated thymidine incorporation in response to stimulation with neisserial proteins compared to irrelevant antigens. Such an irrelevant antigen may be tetanus toxoid. The phenotype of the T-cell lines and clones are preferably also assessed using flow cytometry and specific monoclonal antibodies. The antibodies are preferably $CD4^+$, $CD8^+$ and α/β- and γ/δ- T-cell receptor (TCR) specific monoclonal antibodies.

In a second aspect the present invention provides a method of detecting $CD4^+$ T-cell stimulating proteins, the method comprising fractionating neisserial proteins and testing the ability of said proteins to stimulate proliferation of T-cell lines and clones.

Preferably the T-cell lines and clones are Neisseria specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

The proteins may be fractionated by SDS-PAGE. The fractions are preferably tested for their ability to stimulate the individual T-cell lines and clones. Preferably fractions containing T-cell stimulants are further characterised by SDS-PAGE.

Polyclonal antibodies may be raised to the T-cell stimulating fraction proteins. The antibodies are preferably used to screen a genomic meningococcal or gonococcal expression library. Preferably the expression library is a λZapII library. Isolated neisserial polypeptides which react with the antibodies and their respective DNA fragments are preferably further characterised and sequenced.

In a third aspect, the present invention provides a method of detecting $CD4^+$ T-cell stimulating recombinant proteins, the method comprising screening a genomic meningococcal or gonococcal expression library for recombinant proteins which stimulate T-cell lines and clones.

Preferably the T-cell lines and clones are meningococcal and/or gonococcal specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

Preferably the genomic meningococcal or gonococcal expression library is a λZapII phage library expressing genomic DNA extracted from a strain of *Neisseria meningitidis* or a strain of *Neisseria gonorrhoea.* Preferably a representative pool of recombinant pBluescript SKII plasmid are excised from the phage library and transformed into *E.coli* strain XL1-Blue. Preferably the plasmids are excised into XL1-Blue using a helper phage.

The transformed *E.coli* are preferably cultured in a medium which may contain ampicillin. Meningococcal or gonococcal protein expression is preferably induced by isopropyl-b-D-thio-galactoside.

Preferably the bacteria are heat-killed and sonicated before adding to antigen presenting cells. The expressed proteins are preferably tested for their ability to stimulate the individual T-cell lines and clones. Preferably $CD4^+$ T-cell stimulating cultures are identified and subcultured. The subcultures are preferably rescreened for T-cell stimulation.

Preferably the $CD4^+$ T-cell stimulants are identified by sequencing and may be further characterised.

Alternatively the genomic meningococcal or gonococcal expression library is a λZapII phage library expressing genomic DNA extracted from a meningococcal or gonococcal genomic lambda phage display library.

In a fourth aspect the present invention provides a method of detecting $CD4^+$ T-cell stimulating peptides, the method comprising screening meningococcal or gonococcal genomic phage display libraries (PDLs) to identify peptides which stimulate T-cell lines and clones.

Preferably the T-cell lines and clones are meningococcal and/or gonococcal specific T-cell lines and clones generated according to the method of the first aspect of the invention, as set out above.

Preferably the genomic phage display library (PDL) is generated by fragmenting bacterial DNA, cloning and packaging into bacteriophage vectors. Preferably two vectors are used. The first vector preferably displays peptides up to 1200 amino acids which are expressed at low copy numbers. The second vector preferably displays up to 415 copies of a peptide up to 50 amino acids in size.

Preferably the PDLs are amplified in respective *E.coli* hosts. The cells are preferably heat killed before testing for the ability of the peptides to stimulate the T-cell lines and clones.

Preferably $CD4^+$ T-cell stimulating cultures are identified and subcultured. The subcultures are preferably rescreened for T-cell stimulation.

Preferably the $CD4^+$ T-cell stimulants are identified by sequencing and may be further characterised.

In a fifth aspect the present invention provides a method of detecting $CD4^+$ T-cell stimulating recombinant proteins, using a meningococcal or gonococcal genomic lambda phage display library in accordance with the third aspect of the invention, as set out above.

The meningococcal or gonococcal genomic lambda phage display library is preferably constructed by cloning randomly amplified PCR products using

two random primers, each tagged at 5' end to restriction sites, inserting same into a pre-digested vector, and plating by infecting *E.coli.*

Preferably the vector is a lambda phage and is preferably λpRH825 vector. The amplified and digested DNA fragments are preferably packaged into the lambda phage using a lambda phage packaging kit. Preferably the restriction sites are SpeI or NotI.

Preferably the DNA inserts in the plaques formed are sequenced, thereby confirming that the plaques contain DNA fragments of meningococcal or gonococcal origin.

In a sixth aspect the present invention provides the use of a polypeptide in the manufacture of a vaccine against neisserial disease, the peptide comprising an amino acid sequence as shown in SEQIDNO1 and SEQIDNO2 or an active derivative thereof.

Preferably the polypeptide is a $CD4^+$ T-cell stimulant.

In a seventh aspect the present invention provides the use of a polypeptide in the manufacture of a vaccine against neisserial disease, the peptide comprising an amino acid sequence as shown in SEQIDNO3 and SEQIDNO4 or an active derivative thereof.

Preferably the polypeptide is a $CD4^+$ T-cell stimulant.

In an eighth aspect the invention provides a composition for use as a vaccine against neisserial disease, the composition comprising two peptides with the amino acid sequences as shown in SEQIDNO1 and SEQIDNO2, and SEQIDNO3 and SEQIDNO4 or active derivatives thereof.

In a ninth aspect of the present invention there is provided a nucleotide

sequence comprising a base sequence as shown in SEQIDNO1, or an active derivative thereof, the sequence coding for a polypeptide having an amino acid sequence as shown in SEQIDNO1 and SEQIDNO2, or an active derivative thereof.

In a tenth aspect of the present invention there is provided a nucleotide sequence comprising a base sequence as shown in SEQIDNO3, or an active derivative thereof, the sequence coding for a polypeptide having an amino acid sequence as shown in SEQIDNO3 and SEQIDNO4, or an active derivative thereof.

Embodiments of the invention will now be described by way of example only and with reference to the accompanying drawings and sequences, in which:-

Fig. 1 is a graph illustrating the proliferation responses of peripheral blood lymphocytes (PBLs) of three patients and a healthy donor to meningococcal proteins.

Fig. 2 is a graph illustrating the proliferation indices of a T-cell line with fraction (SI-V) of meningococcal proteins separated by SDS PAGE.

Fig. 3 is a graph illustrating the proliferation indices of a T-cell line to subfractions A, B, C and D of section SI in Fig. 2, and also the proliferation index of concanavalin A (Con A) and whole cell lysate of iron-depleted meningococci (SD-).

SEQIDNO1 shows the nucleotide base sequence and the corresponding amino acid sequence of a gene and a polypeptide encoded thereby, according to one aspect of the present invention;

SEQIDNO2 shows the polypeptide sequence of SEQIDNO1;

SEQIDNO3 shows the nucleotide base sequence and the corresponding amino acid sequence of a gene and a polypeptide encoded thereby, according to another aspect of the present invention; and

SEQIDNO4 shows the polypeptide sequence of SEQIDNO3.

In order to identify meningococcal $CD4^+$ T-cell-stimulating peptides we adopted a number of different programmes all of which involve screening meningococcal peptide antigens, using meningococcal-specific $CD4^+$ T-cell lines and clones. These lines and clones have been generated over the past three years or so, from the peripheral blood of normal donors and patients recovering from invasive meningococcal disease. In Nottingham, we are in a unique and ideal position to study *in-vivo* primed human T-cells obtained from naturally infected patients, with fresh peripheral blood samples obtained from patients at different stages of illness, namely the acute stage (on admission), early convalescence (seven days after admission), late convalescence (six weeks after discharge) and after full recovery (3 months and twelve months after discharge). We have already generated T-cell lines and clones, specific to meningococcal proteins from the peripheral blood of eight patients recovering from meningococcal disease (P1-8) and eleven healthy donors (D1-11). The healthy donors were identified among twenty five volunteers by testing their peripheral blood lymphocyte (PBL) proliferation in response to meningococcal proteins.

Lymphocyte proliferation assays:

Briefly, PBLs were isolated from heparinised blood samples by centrifugation over Histopaque (Sigma). The PBLs were washed and cultured in 96-well tissue culture plates at 2×10^5 cells/well in RPMI medium containing 10% human AB serum (RPMI-AB). Meningococcal proteins (from strain SD, B:15:P1,16) were prepared by growing the organism under iron restriction, to induce the expression of iron-regulated proteins which are also expressed *in vivo* [Ala'Aldeen, 1994]. The meningococcal proteins (SD-), antigens from *Candida albicans* (a recall antigen) or phytohaemagglutinin (PHA, positive control) were added to quadruplicate wells. RPMI-AB alone (with no antigen) was added to quadruplicate wells to serve as the background. After five days all cultures were pulsed with 1μCi of tritiated thymidine and incorporation of thymidine was determined after another eighteen hours. A positive response was defined as a PBL proliferation index of at least 2 (see Fig. 1).

Continuous T-cell lines were established by culturing PBLs with the meningococcal proteins and Interleukin 2 (IL-2) for five days, and activated T-cell blasts were stimulated to proliferate by a further nine days culture with IL-2 only. The lines were then maintained by weekly stimulation with proteins in the presence of feeder cells and IL-2. Autologous Epstein-Barr virus transformed B-lymphocytes (EBVB) were used as antigen-presenting feeder cells following irradiation (6000R).

T-cell clones are defined here as the population of cells which originate from a single T-cell. Single T-cell receptors (TCRs) can engage with an extraordinary small number of peptide-HLA complexes (<10/cell) [Valitute, 1995], therefore T-cell clones will provide a highly sensitive system by which it will be possible to detect the presence of peptide antigens within mixtures of proteins. T-cell lines, specific to meningococcal antigens, were seeded at 0.3 cell/well in 96-well tissue culture plates in the presence of irradiated (non-proliferating) autologous EBVB feeder cells, plus low doses of IL-2 [Sinigaglia, 1991]. Cell growth was detected microscopically after one-two weeks and growing cells expanded further by stimulation with meningococcal proteins. All T-cell lines and clones were assessed for the phenotype (and ascertained to be $CD4^+$ T-cells), using flow cytometry and CD4, CD8 and α/β- and γ/δ- TCR-specific monoclonal antibodies. Their specificity to meningococcal proteins were tested by measurement of tritiated thymidine incorporation in response to stimulation with meningococcal proteins compared to irrelevant antigens e.g. tetanus toxoid. Large numbers T-cell lines, oligoclones and clones from patients and normal donors have been identified and stored in liquid nitrogen until further use.

T-cell responses to fractionated meningococcal proteins

Meningococcal proteins were fractionated according to their molecular weights by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Two methods were used to prepare the separated proteins for addition to the T-cell cultures:

a) Fractionated proteins were transferred onto nitrocellulose membranes which were transversely divided into five equal sections labelled SI-V, containing proteins of approximate molecular weight range >130 kDa, 70-130 kDa, 50-70 kDa, 34-50 kDa and <34 kDa, respectively. Membranes were then solubilised with dimethyl sulphoxide and tested for their ability to stimulate T-cells using the established meningococcal specific T-cell lines. Using one of the cell lines, section SI (which contained proteins >130 kDa) caused greater T-cell proliferation than any of the other sections (Fig. 2). T-cell lines fed with either EBV-B-cells or fresh autologous PBLs consistently gave similar results.

b) In the second method, SDS-gels containing the fractionated proteins were cut into transverse sections corresponding to the five fractions obtained by the nitrocellulose membrane method. The proteins were then directly eluted from the gel sections and purified by precipitation with organic solvents. This enabled us to measure protein concentrations in each fraction and confirm that whether differences in protein concentration were not responsible for the differences observed in Figure 2. Equivalent concentrations of purified proteins were used in lymphocyte proliferation assays. The results were consistent with those of the nitrocellulose membrane blot method (not shown).

Section SI consists of more than 12 proteins as seen on silver stained gels, ranging from 130-599 kDa (not shown). Therefore, it was subdivided into four fractions, FIA-D, and their proteins were eluted from gels as described above. The eluted proteins were tested for their ability to stimulate T-cell proliferation. As shown in Figure 3, using T-cell line of a patient, fractions FIC and D induced extremely high T-cell proliferation indices ($\underline{c}$ 30), higher than fractions FIA and FIB, the whole of SI or the total SD-protein preparation. Another T-cell line showed the highest T-cell stimulation indices with fraction FIB and FIC, followed by FID, possibly reflecting the HLA specific response.

FIC was chosen for further characterisation and silver staining of SDS-gels showed that it contains four distinct protein bands (not shown). Rabbit polyclonal antibodies were raised to eluted FIC proteins and used to screen an

already established genomic expression (λ Zap II) library. Several reactive meningococcal polypeptides and their respective DNA fragments were isolated. Two of the most promising ones (TspA and TspB) were further studied. The DNA fragments were sequenced and with help from the Sanger-released genomic sequences which were produced by the Neisseria Meningitidis Sequencing Group at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/AAREADME.release-policy.txt, the genes encoding these two proteins were then constructed (see SEQIDNO1-4) and cloned into high expression vectors.

The exact sequences can vary among different isolates of meningococci due to the nature of the organism and its ability to mutate any gene any time. This is a universal problem inherent with any gene of these Neisseria organisms. Equivalent genes with homologous sequences exist in *Neisseria gonorrhoea*, as detected on the recently released gonococcal genomic sequence data obtained on the Internet from Oklahoma University, U.S.A.

Western blot experiments on TspA and TspB, using human convalescent sera, confirmed that both proteins are expressed in-vivo and stimulate B-cells following natural infection. The cloned proteins also induced strong $CD4^+$ T-cell stimulatory effect in our T-cell proliferation assays. These indicate very clearly that they are promising vaccine candidates.

Finally, fractions FIB and FID, and Section SII and SV which produced net-positive T-cell stimulatory effects may consists of many T-cell stimulatory antigens (Fig. 1 and 3), and will be examined.

Detection of T-cell antigens by phage-expression cloning

Over the past two years or so, we have also established a robust screening system for the identification of $CD4^+$ T-cell stimulating recombinant proteins, using an expression cloning protocol, which involves screening genomic meningococcal expression libraries.

1. λZapII Expression Library

This method had been successfully applied in other organisms to identify helper T-cell epitopes [Sanderson, 1995; Mougneau, 1995]. Briefly, we used an existing a λZapII phage library expressing genomic DNA extracted from strain SD N. *meningitidis* [Palmer, 1993 #214]. The library contains 2 x 10^5 recombinants with an average size of insert of 2.3 kb (range up to 10 kb). A representative pool of recombinant pBluescript SKII plasmid were excised (*in vivo*) from the phage library and transformed into *E. coli* strain XL1-Blue, using ExAssist helper phage (Stratgene) as described previously [Ala'Aldeen, 1996; Palmer, 1993].

Transformed *E. coli* with the pBluescript plasmid carrying meningococcal genes were diluted in selective culture media (containing ampicillin) and put in 96-well microtitre plates at 20-30 transformants/wells. The plates were incubated overnight at 37°C with shaking and replicate cultures were made by splitting the overnight cultures, and the original master plates stored at 4°C. The splits were grown in epindorfs for 2-3 hours in fresh medium to OD_{600}=0.3, then incubated for an additional 2h with 1mM isopropyl-b-D-thio-galactoside (IPTG) to induce meningococcal protein expression. Bacteria were heat-killed, sonicated and added to the antigen presenting cells, and tested for their ability to stimulate individual T-cell lines and clones. Negative controls were sonicates of the same *E. coli* strain transformed with pBluescript SKII with no meningococcal DNA insert. Strong T-cell stimulating wells were identified and their corresponding reference wells diluted and subcultured. Up to 100 single colonies (representing single organisms with single plasmids) were isolated and re-screened for T-cell stimulation. Only potent T-cell stimulants were saved and further pursued. This approach proved highly rewarding, and so far we have identified and further characterised two, previously unknown, potent T-cell stimulating meningococcal polypeptides.

2. T-cell antigen detection using phage display libraries (PDL)

Displaying foreign peptides on the surface of bacteriophages is a relatively new but well-established technology. This is different from the normal phage libraries which carry the cloned genes and express and release the proteins inside a host bacterium and not on their own outer coat. In phage display libraries, displayed peptides are encoded as DNA inserts in the structural gene for one of the viral coat proteins and will then appear on the surface of the phage capsid. There are several phage display systems available, each with specific advantages. For example, some are filamentous and others are lytic, some are used as random display libraries (non-specific) which may be used to detect mimotopes, and others are more specific genomic libraries. It is important to note that most phage display libraries have been probed with antibodies in search of specific peptides. Here, we adopted a highly novel approach involving the use of T-cell lines/clones to screen two different meningococcal genomic PDLs to identify good T-cell stimulating peptides. As far as we are aware, these have not been done before.

a) T7Select1 and T7Select415 PDL

One of the novel lytic bateriophages is Novagen's T7Select Phage Display System which is easy to use and has the capacity to display peptides up to 1200 amino acids, equivalent to 3.6 kb, with protein molecular weight over 100kDa. Such high molecular weight proteins are usually expressed at low copy numbers by T7Select1. Phage T7Select415, however, is capable of displaying up to 415 copies of a peptide up to 50 amino acids in size. Phage assembly occurs in the *E. coli* cytoplasm and mature phages are release by cell lysis. The latter process occurs within a few hours of infection, which makes the system very rapid. To create a genomic display library, meningococcal DNA will be fragmented to appropriate sizes and cloned and packaged into both T7Select1 and T7Select415 vectors as described in the Novagen's T7Select System manual [Novagen, 1996]. This dual approach should allow us to screen for both large and small polypeptides.

A representative population of these PDLs expressing meningococcal

proteins will be diluted and distributed as oligoclones into 96-well microtitre plates. To each well, appropriate *E. coli* host strains (BL,21 for T7Select415 and BLT5403 for T7Select1) will be added to amplify the diluted phage population in these wells. The plates will be split into identical duplicates, one of which will be stored as the reference, and the other heat-killed and tested for the ability to stimulate the T-cell lines/clones as described above for the λZAPII library.

b) λpRH825 random meningococcal epitope display library

Another system to utilise is the display of proteins and small peptides on a modified lambda capsid protein D. This protein, which is of 11 kDa with 405 copies expressed as trimers on the phage head [Sternberg, 1995; Mikawa, 1996], is capable of an efficient display of foreign peptides that are fused to its amino- or carboxy-termini [Mikawa, 1996]. This system was successfully used to display a Hepatitis C genomic cDNA library [Alter, 1995] and, more recently, to generate a randomly amplified genomic PDL of known organisms [Lambert, 1993; Kwong-Kowk, 1996; Tomei, 1993]. This involves generating randomly amplified DNA fragments of a known DNA template, using short (random) oligonucleotide primers in polymerase chain reaction (PCR). In collaboration with the Instituto do Ricerche di Biologia Molcculare (IRBM) in Rome, Italy, we have recently constructed a meningococcal genomic lambda phage display library by cloning randomly amplified PCR products into λpRH825 vector, using two random primers, each tagged at 5' end to SpeI or NotI restriction sites to facilitate insertion into the predigested vector. Packaging amplified and digested DNA fragments into lambda phage was performed using a lambda packaging kit (Pharmacia Biotech) and plated by infection of the *E. coli* strain BB4. This yielded 5 x 10^7 plaques, of which a sample of 100 pfu were randomly chosen, and their DNA inserts sequenced. Sequence alignment of the obtained sequence data with those available for *N. Meningitidis* (Sanger, Wellcome) and/or *N. Gonorrhoea*, confirmed that all the chosen plaques contained DNA fragments of meningococcal origin. The fragment sizes ranged from 100-200 bp, representing deduced peptides of up to 60 amino acids long. This PDL was

prepared and established in IRBM and is now ready to be used for the identification of $CD4^+$ T-cell stimulating recombinant peptides, using the same cloning technique described for the λZapII phage system.

We have adopted several selection criteria to focus the search on relevant, potent and promiscuous T-cell epitopes.

Initially, only candidate peptides, which are likely to contain multiple T-cell epitopes that are immunogenic for $CD4^+$ Th-cells (not $CD8^+$ T-cells) and presented on MHC class II (HLA-DR, DQ or DP in humans) are studied. Only T-helper (Th) antigens, that bind to a number of widely ranging HLA-types, will be selected. It will be determined whether each patient's $CD4^+$ Th-response to a candidate meningococcal peptide is due to an established memory Th population (CD45RO+) or to activation of naive T-cells (CD45RA+). Peptide candidates which activate either the Th2 subset of $CD4^+$ T-cell or the Th1 subset will be selected. The therapeutic efficacy of both Th1 and Th2-inducing candidate peptides will be evaluated. T-cell clones specific for candidate antigens will be amplified and used to identify the individual T-cell epitopes.

In order to identify and then characterise core epitopes of each candidate peptide, progressively smaller fragments of the DNA will be cloned, expressed and further examined for T-cell stimulation. To define epitopes more accurately, short overlapping peptides representing the defined T-cell stimulating subunits will be synthesised and re-examined. Then N- and C-terminal truncated analogs of the most immunogenic peptide fragment will be synthesised and tested likewise. Finally, alanine scanning mutational analysis will be employed to identify critical amino acid positions responsible for both TCR contact and HLA-class II contact. Here, a series of peptide analogs of the core epitope identified after N- and C-terminal truncation are synthesised, each with a single alanine substituted at successive amino acid positions, and effects on T-cell immunogenicity and on HLA-binding are assessed [Nelson, 1996]. The isotype of class II HLA molecule restriction specificity will be identified for each T-cell clone by antibody blocking experiments.

As a part of the characterisation of the identified proteins, we will study the diversity of these proteins among various strains of meningococci. We have a large collection of clinical isolates of meningococci, the proteins of these strains will be purified (from the gels or clones), tested for T-cell stimulatory capacity and characterised in a way similar to that used for strain SD. Proteins that are expressed in all or more of these stains will be focused on.

Identification of HLA restriction

To determine whether different HLA class II molecules present different parts of individual proteins, one of two methods will be used. The protein sub-fragments and their overlapping peptides described above will be tested for their capacity to stimulate T-cell clones generated from different individuals (volunteers or patients). Alternatively, lymphocyte donors will be HLA typed, and the association of responsiveness to particular proteins (or epitopes) and certain alleles of HLA-DR, -DQ or -DP will be determined.

A central aim is to identify T-cell immunogens of *N. meningitidis* which will stimulate T-cell help for the production of protective anti- meningococcal antibodies. Having identified dominant T-cell antigens amongst the proteins, their ability to stimulate T-cell help for antibody production will be investigated *in vivo* in animals and in an *in vitro* immunisation system which has been established and optimised in our laboratories [Davenport, 1992]. Protein fragments or peptides that stimulate T-cells from individuals covering a range of HLA types will be studies for the presence of B-cell epitopes. If the protein contains B-cell epitopes than antibodies from individuals naturally immune to meningococcal disease should recognise these proteins in immunoblots or ELISA. If no B-cell epitopes are recognised then the identified T-cell epitopes will be conjugated to previously characterised B-cell immunogens such as the meningococcal capsular polysaccharides, the class (1, 2/3) proteins, the transferrin binding proteins ... etc.

Whilst endeavouring in the foregoing specification to draw attention to

the features of the invention believed to be of particular importance it should be understood that the Applicant claims protection in respect of any patentable feature hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.

SEQUENCE LISTING

(1) Information for SEQIDNO1:

(a) Sequence Characteristics:

(i) Length : 2761 base pairs

(ii) Type : Nucleic acid

(iii) Strandedness : Double

(b) Molecule type : DNA (genomic)

(c) Original Source:

(i) Organism : *Neisseria meningitidis*

(ii) Strain : SD, serogroup B (B:15:Pl.16)

(2) Information for SEQIDNO2:

(a) Sequence Characteristics:

(i) Length : 880 amino acids

(ii) Type : amino acid

(iii) Topology : linear

(b) Molecule type : protein

(c) Original Source:

(i) Organism : *Neisseria meningitidis*

(ii) Strain : SD, serogroup B (B:15:Pl.16)

(3) Information for SEQIDNO3:

(a) Sequence Characteristics:

(i) Length : 1639 base pairs

(ii) Type : Nucleic acid

(iii) Strandedness : Double

(b) Molecule type : DNA (genomic)

(c) Original Source:

(i) Organism : *Neisseria meningitidis*

(ii) Strain : SD, serogroup B (B:15:Pl.16)

(4) Information for SEQIDNO4:

(a) Sequence Characteristics:

(i) Length : 504 amino acids

(ii) Type : amino acid

(iii) Topology : linear

(b) Molecule type : protein

(c) Original Source:

(i) Organism : *Neisseria meningitidis*

(ii) Strain : SD, serogroup B (B:15:Pl.16)

SEQ ID NO 1

```
                                                    GATTGATG  CAAATATGCA   18
CAGATGTTTT  TGAAAAAAGA  TGGAGATATG  TCATAATTTG  TAAAAACGGC   68
GAATGATTGC  CGTTTAAAAT  GTGGCGGCGG  TCGGTACATT  CACATATTAA  118

ATG CCC GCC GGC CGA CTG CCC CGC CGA TGC CCG ATG ATG ACG AAA 163
Met Pro Ala Gly Arg Leu Pro Arg Arg Cys Pro Met Met Thr Lys
                                -10                 -15
1               -5

TTT ACA GAC TGT ACG CGG TCA AAC CGT ATT CAG CCG CCA ACC CAC 208
Phe Thr Asp Cys Thr Arg Ser Asn Arg Ile Gln Pro Pro Thr His
            -20                 -25                 -30

AGG GGA TAC ATC TTG AAA AAC AAC AGA CAA ATC AAA CTG ATT GCC 253
Arg Gly Tyr Ile Leu Lys Asn Asn Arg Gln Ile Lys Leu Ile Ala
            -35                 -40                 -45

GCC TCC GTC GCA GTT GCC GCA TCC TTT CAG GCA CAT GCT GGA CTG 298
Ala Ser Val Ala Val Ala Ala Ser Phe Gln Ala His Ala Gly Leu
            -50                 -55                 -60

GGC GGA CTG AAT ATC CAG TCC AAC CTT GAC GAA CCC TTT TCC GGC 343
Gly Gly Leu Asn Ile Gln Ser Asn Leu Asp Glu Pro Phe Ser Gly
            -65                 -70                 -75

AGC ATT ACC GTA ACC GGC GAA GAA GCC AAA GCC CTG CTA GGC GGC 388
Ser Ile Thr Val Thr Gly Glu Glu Ala Lys Ala Leu Leu Gly Gly
            -80                 -85                 -90

GGC AGC GTT ACC GTT TCC GAA AAA GGC CTG ACC GCC AAA GTC CAC 433
Gly Ser Val Thr Val Ser Glu Lys Gly Leu Thr Ala Lys Val His
            -95                 100                 105

AAG TTG GGC GAC AAA GCC GTC ATT GCC GTT TCT TCC GAA CAG GCA 478
Lys Leu Gly Asp Lys Ala Val Ile Ala Val Ser Ser Glu Gln Ala
            110                 115                 120

GTC CGC GAT CCC GTC CTG GTA TTC CGC ATC GGC GCA GGC GCA CAG 524
Val Arg Asp Pro Val Leu Val Phe Arg Ile Gly Ala Gly Ala Gln
            125                 130                 135

GTA CGC GAA TAC ACC GCC ATC CTC GAT CCT GTC GGC TAC TCG CCC 568
Val Arg Glu Tyr Thr Ala Ile Leu Asp Pro Val Gly Tyr Ser Pro
            140                 145                 150

AAA ACC AAA TCT GCA CTT TCA GAC GGC AAG ACA CAC CGC AAA ACC 613
Lys Thr Lys Ser Ala Leu Ser Asp Gly Lys Thr His Arg Lys Thr
            155                 160                 165

GCT CCG ACA GCA GAG TCC CAA GAA AAT CAA AAC GCC AAA GCC CTC 658
Ala Pro Thr Ala Glu Ser Gln Glu Asn Gln Asn Ala Lys Ala Leu
            170                 175                 180

CGC AAA ACC GAT AAA AAA GAC AGC GCG AAC GCA GCC GTC AAA CCG 703
Arg Lys Thr Asp Lys Lys Asp Ser Ala Asn Ala Ala Val Lys Pro
            185                 190                 195

GCG TAC AAC GGC AAA ACC CAT ACC GTC CGC AAA GGC GAA ACG GTC 748
Ala Tyr Asn Gly Lys Thr His Thr Val Arg Lys Gly Glu Thr Val
            200                 205                 210

AAA CAG ATT GCC GCC GCC ATC CGC CCG AAA CAC CTG ACG CTC GAA 793
Lys Gln Ile Ala Ala Ala Ile Arg Pro Lys His Leu Thr Leu Glu
            215                 220                 225

CAG GTT GCC GAT GCG CTG CTG AAG GCA AAC CCA AAT GTT TCC GCA 838
Gln Val Ala Asp Ala Leu Leu Lys Ala Asn Pro Asn Val Ser Ala
            230                 235                 240

CAC GGC AGA CTG CGT GCG GGC AGC GTG CTT CAC ATT CCG AAT CTG 883
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His Gly Arg Leu Arg     Gly Ser Val Leu His Ile Pro [redacted] Leu
                245                 250                 255

AAC AGG ATC AAA GCG GAA CAA CCC AAA CCG CAA ACG GCG AAA CCC 928
Asn Arg Ile Lys Ala Glu Gln Pro Lys Pro Gln Thr Ala Lys Pro
                260                 265                 270

AAA GCC GAA ACC GCA TCC ATG CCG TCC GAA CCG TCC AAA CAG GCA 973
Lys Ala Glu Thr Ala Ser Met Pro Ser Glu Pro Ser Lys Gln Ala
                275                 280                 285

ACG GTA GAG AAA CCG GTT GAA AAA CCT GAA GCA AAA GTT GCC GCG 1018
Thr Val Glu Lys Pro Val Glu Lys Pro Glu Ala Lys Val Ala Ala
                290                 295                 300

CCC GAA GCA AAA GCG GAA AAA CCG GCC GTT CGA CCC GAA CCT GTA 1063
Pro Glu Ala Lys Ala Glu Lys Pro Ala Val Arg Pro Glu Pro Val
                305                 310                 315

CCC GCT GCA AAT ACT GCC GCA TCG GAA ACC GCT GCC GAA TCC GCC 1108
Pro Ala Ala Asn Thr Ala Ala Ser Glu Thr Ala Ala Glu Ser Ala
                320                 325                 330

CCC CAA GAA GCC GCC GCT TCT GCC ATC GAC ACG CCG ACC GAC GAA 1153
Pro Gln Glu Ala Ala Ala Ser Ala Ile Asp Thr Pro Thr Asp Glu
                335                 340                 345

ACC GGT AAC GCC GTT TCC GAA CCT GTC GAA CAG GTT TCT GCC GAA 1198
Thr Gly Asn Ala Val Ser Glu Pro Val Glu Gln Val Ser Ala Glu
                350                 355                 360

GAA GAA ACC GAA AGC GGA CTG TTC GGC GGT TCG TAC ACC TTG CTG 1243
Glu Glu Thr Glu Ser Gly Leu Phe Gly Gly Ser Tyr Thr Leu Leu
                365                 370                 375

CTT GCC GGC GGA GGC GCG GCA TTG ATC GCC CTG CTG CTG CTT TTG 1288
Leu Ala Gly Gly Gly Ala Ala Leu Ile Ala Leu Leu Leu Leu Leu
                380                 385                 390

CGC CTT GCC CAA TCC AAA CGC GCG CGC CGT ACC GAA GAA TCC GTC 1333
Arg Leu Ala Gln Ser Lys Arg Ala Arg Arg Thr Glu Glu Ser Val
                395                 400                 405

CCT GAG GAA GAG CCT GAC CTT GAC GAC GCG GCA GAC GAC GGC ATA 1378
Pro Glu Glu Glu Pro Asp Leu Asp Asp Ala Ala Asp Asp Gly Ile
                410                 415                 420

GAA ATC ACC TTT GCC GAA GTC GAA ACT CCG GCA ACG CCC GAA CCC 1423
Glu Ile Thr Phe Ala Glu Val Glu Thr Pro Ala Thr Pro Glu Pro
                425                 430                 435

GCT CCG AAA AAC GAT GTA AAC GAC ACA CTT GCC TTA GAT GGG GAA 1468
Ala Pro Lys Asn Asp Val Asn Asp Thr Leu Ala Leu Asp Gly Glu
                440                 445                 450

TCT GAA GAA GAG TTG TCG GCA AAA CAA ACG TTC GAT GTC GAA ACC 1513
Ser Glu Glu Glu Leu Ser Ala Lys Gln Thr Phe Asp Val Glu Thr
                455                 460                 465

GAT ACG CCT TCC AAC CGC ATC GAC TTG GAT TTC GAC AGC CTG GCA 1558
Asp Thr Pro Ser Asn Arg Ile Asp Leu Asp Phe Asp Ser Leu Ala
                470                 475                 480

GCC GCG CAA AAC GGC ATT TTA TCC GGC GCA CTT ACG CAG GAT GAA 1603
Ala Ala Gln Asn Gly Ile Leu Ser Gly Ala Leu Thr Gln Asp Glu
                485                 490                 495

GAA ACC CAA AAA CGC GCG GAT GCC GAT TGG AAC GCC ATC GAA TCC 1648
Glu Thr Gln Lys Arg Ala Asp Ala Asp Trp Asn Ala Ile Glu Ser
                500                 505                 510
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ACA GAC AGC GTG TAC GAA CCC GAG ACC TTC AAC CCG TAC AAC CCT 1693
Thr Asp Ser Val Tyr Glu Pro Glu Thr Phe Asn Pro Tyr Asn Pro
515 520 525

GTC GAA ATC GTC ATC GAC ACG CCC GAA CCG GAA TCT GTC GCC CAA 1738
Val Glu Ile Val Ile Asp Thr Pro Glu Pro Glu Ser Val Ala Gln
530 535 540

ACT GCC GAA AAC AAA CCG GAA ACC GTC GAT ACC GAT TTC TCG GAC 1783
Thr Ala Glu Asn Lys Pro Glu Thr Val Asp Thr Asp Phe Ser Asp
545 550 555

AAC CTG CCC TCA AAC AAC CAT ATC GGC ACA GAA GAA ACA GCT TCC 1828
Asn Leu Pro Ser Asn Asn His Ile Gly Thr Glu Glu Thr Ala Ser
560 565 570

GCA AAA CCT GCC TCA CCC TCC GGA CTG GCA GGC TTC CTG AAG GCT 1873
Ala Lys Pro Ala Ser Pro Ser Gly Leu Ala Gly Phe Leu Lys Ala
575 580 585

TCC TCG CCC GAA ACC ATC TTG GAA AAA ACA GTT GCC GAA GTC CAA 1918
Ser Ser Pro Glu Thr Ile Leu Glu Lys Thr Val Ala Glu Val Gln
590 595 600

ACA CCG GAA GAG TTG CAC GAT TTC CTG AAA GTG TAC GAA ACC GAT 1963
Thr Pro Glu Glu Leu His Asp Phe Leu Lys Val Tyr Glu Thr Asp
605 610 615

GCC GTC GCG GAA ACT GCG CCT GAA ACG CCC GAT TTC AAC GCC GCC 2008
Ala Val Ala Glu Thr Ala Pro Glu Thr Pro Asp Phe Asn Ala Ala
620 625 630

GCA GAC GAT TTG TCC GCA TTG CTT CAA CCT GCC GAA GCA CCG TCC 2053
Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ser
635 640 645

GTT GAG GAA AAT ATA ACG GAA ACC GTT GCC GAA ACA CCC GAC TTC 2098
Val Glu Glu Asn Ile Thr Glu Thr Val Ala Glu Thr Pro Asp Phe
650 655 660

AAC GCC ACC GCA GAC GAT TTG TCC GCA TTA CTT CAA CCT TCT GAA 2143
Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu
665 670 675

GTA CCT GCC GTT GAG GAA AAT GCA GCG GAA ATC GTT GCC GAT GAT 2188
Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Val Ala Asp Asp
680 685 690

TTG TCC GCA CTG TTG CAA CCT GCT GAA GCA CCG GCT GTT GAG GAA 2233
Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ala Val Glu Glu
695 700 705

AAT GTA ACG GAA ACT GTT GCC GAA ACG TCC GAC TTC CAC ACC GCC 2278
Asn Val Thr Glu Thr Val Ala Glu Thr Ser Asp Phe His Thr Ala
710 715 720

GCA GAC GAT TTG TCC GCA CTG TTG CAA CCT GCT GAA GTA CCG GCC 2323
Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Val Pro Ala
725 730 735

GTT GAG GAA AAT GTA ACG AAA ACC GTT GCC GAA ATA CCT GAT TTC 2368
Val Glu Glu Asn Val Thr Lys Thr Val Ala Glu Ile Pro Asp Phe
740 745 750

AAC GCC ACC GCA GAC GAT TTG TCC GCA TTA CTT CAA CCT TCT GAA 2413
Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu
755 760 765

GTA CCG GCC GTT GAG GAA AAT GCA GCG GAA ATC ACT TTG GAA ACG 2458
Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Thr Leu Glu Thr
770 775 780

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CCT GAT TCC AAC ACC TC[redacted]GAG GCA GAC GCT TTG CCC GAC TT[redacted]TG 2503
Pro Asp Ser Asn Thr Ser Glu Ala Asp Ala Leu Pro Asp Phe Leu
                785                 790                 795

AAA GAC GGC GAG GAG GAA ACG GTA GAT TGG AGC ATC TAC CTC TCG 2548
Lys Asp Gly Glu Glu Glu Thr Val Asp Trp Ser Ile Tyr Leu Ser
                800                 805                 810

GAA GAA AAT ATC CCA AAT AAT GCA GAT ACC AGT TTC CCT TCG GAA 2593
Glu Glu Asn Ile Pro Asn Asn Ala Asp Thr Ser Phe Pro Ser Glu
                815                 820                 825

TCT GTA GGT TCT GAC GCG CCT TCC GAA GCG AAA TAC GAC CTT GCC 2638
Ser Val Gly Ser Asp Ala Pro Ser Glu Ala Lys Tyr Asp Leu Ala
                830                 835                 840

GAA ATG TAT CTC GAA ATC GGC GAC CGC GAT GCC GCT GCC GAG ACA 2683
Glu Met Tyr Leu Glu Ile Gly Asp Arg Asp Ala Ala Ala Glu Thr
                845                 850                 855

GTG CAG AAA TTG CTG GAA GAA GCG GAA GGC GAC GTA CTC AAA CGT 2728
Val Gln Lys Leu Leu Glu Glu Ala Glu Gly Asp Val Leu Lys Arg
                860                 865                 870

GCC CAA GCA TTG GCG CAG GAA TTG GGT ATT TGA              2761
Ala Gln Ala Leu Ala Gln Glu Leu Gly Ile Stop
                875                 880
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SEQ ID NO 2

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Met Pro Ala Gly Arg Leu Pro Arg Arg Cys Pro Met Met Thr Lys
1               -5                  -10                 -15

Phe Thr Asp Cys Thr Arg Ser Asn Arg Ile Gln Pro Pro Thr His
                -20                 -25                 -30

Arg Gly Tyr Ile Leu Lys Asn Asn Arg Gln Ile Lys Leu Ile Ala
                -35                 -40                 -45

Ala Ser Val Ala Val Ala Ala Ser Phe Gln Ala His Ala Gly Leu
                -50                 -55                 -60

Gly Gly Leu Asn Ile Gln Ser Asn Leu Asp Glu Pro Phe Ser Gly
                -65                 -70                 -75

Ser Ile Thr Val Thr Gly Glu Glu Ala Lys Ala Leu Leu Gly Gly
                -80                 -85                 -90

Gly Ser Val Thr Val Ser Glu Lys Gly Leu Thr Ala Lys Val His
                -95                 100                 105

Lys Leu Gly Asp Lys Ala Val Ile Ala Val Ser Ser Glu Gln Ala
                110                 115                 120

Val Arg Asp Pro Val Leu Val Phe Arg Ile Gly Ala Gly Ala Gln
                125                 130                 135

Val Arg Glu Tyr Thr Ala Ile Leu Asp Pro Val Gly Tyr Ser Pro
                140                 145                 150

Lys Thr Lys Ser Ala Leu Ser Asp Gly Lys Thr His Arg Lys Thr
                155                 160                 165

Ala Pro Thr Ala Glu Ser Gln Glu Asn Gln Asn Ala Lys Ala Leu
                170                 175                 180

Arg Lys Thr Asp Lys Lys Asp Ser Ala Asn Ala Ala Val Lys Pro
                185                 190                 195

Ala Tyr Asn Gly Lys Thr His Thr Val Arg Lys Gly Glu Thr Val
                200                 205                 210

Lys Gln Ile Ala Ala Ala Ile Arg Pro Lys His Leu Thr Leu Glu
                215                 220                 225

Gln Val Ala Asp Ala Leu Leu Lys Ala Asn Pro Asn Val Ser Ala
                230                 235                 240

His Gly Arg Leu Arg Ala Gly Ser Val Leu His Ile Pro Asn Leu
                245                 250                 255

Asn Arg Ile Lys Ala Glu Gln Pro Lys Pro Gln Thr Ala Lys Pro
                260                 265                 270

Lys Ala Glu Thr Ala Ser Met Pro Ser Glu Pro Ser Lys Gln Ala
                275                 280                 285

Thr Val Glu Lys Pro Val Glu Lys Pro Glu Ala Lys Val Ala Ala
                290                 295                 300

Pro Glu Ala Lys Ala Glu Lys Pro Ala Val Arg Pro Glu Pro Val
                305                 310                 315

Pro Ala Ala Asn Thr Ala Ala Ser Glu Thr Ala Ala Glu Ser Ala
                320                 325                 330

Pro Gln Glu Ala Ala Ala Ser Ala Ile Asp Thr Pro Thr Asp Glu
                335                 340                 345
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Thr Gly Asn Ala Val S[redacted]Glu Pro Val Glu Gln Val Ser A[redacted]Glu
350 355 360

Glu Glu Thr Glu Ser Gly Leu Phe Gly Gly Ser Tyr Thr Leu Leu
365 370 375

Leu Ala Gly Gly Gly Ala Ala Leu Ile Ala Leu Leu Leu Leu Leu
380 385 390

Arg Leu Ala Gln Ser Lys Arg Ala Arg Arg Thr Glu Glu Ser Val
395 400 405

Pro Glu Glu Glu Pro Asp Leu Asp Asp Ala Ala Asp Asp Gly Ile
410 415 420

Glu Ile Thr Phe Ala Glu Val Glu Thr Pro Ala Thr Pro Glu Pro
425 430 435

Ala Pro Lys Asn Asp Val Asn Asp Thr Leu Ala Leu Asp Gly Glu
440 445 450

Ser Glu Glu Glu Leu Ser Ala Lys Gln Thr Phe Asp Val Glu Thr
455 460 465

Asp Thr Pro Ser Asn Arg Ile Asp Leu Asp Phe Asp Ser Leu Ala
470 475 480

Ala Ala Gln Asn Gly Ile Leu Ser Gly Ala Leu Thr Gln Asp Glu
485 490 495

Glu Thr Gln Lys Arg Ala Asp Ala Asp Trp Asn Ala Ile Glu Ser
500 505 510

Thr Asp Ser Val Tyr Glu Pro Glu Thr Phe Asn Pro Tyr Asn Pro
515 520 525

Val Glu Ile Val Ile Asp Thr Pro Glu Pro Glu Ser Val Ala Gln
530 535 540

Thr Ala Glu Asn Lys Pro Glu Thr Val Asp Thr Asp Phe Ser Asp
545 550 555

Asn Leu Pro Ser Asn Asn His Ile Gly Thr Glu Glu Thr Ala Ser
560 565 570

Ala Lys Pro Ala Ser Pro Ser Gly Leu Ala Gly Phe Leu Lys Ala
575 580 585

Ser Ser Pro Glu Thr Ile Leu Glu Lys Thr Val Ala Glu Val Gln
590 595 600

Thr Pro Glu Glu Leu His Asp Phe Leu Lys Val Tyr Glu Thr Asp
605 610 615

Ala Val Ala Glu Thr Ala Pro Glu Thr Pro Asp Phe Asn Ala Ala
620 625 630

Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ser
635 640 645

Val Glu Glu Asn Ile Thr Glu Thr Val Ala Glu Thr Pro Asp Phe
650 655 660

Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu
665 670 675

Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Val Ala Asp Asp
680 685 690

Leu Ser Ala Leu Leu Gln Pro Ala Glu Ala Pro Ala Val Glu Glu

695 700

sn Val Thr Glu Thr Val Ala Glu Thr Ser Asp Phe His Thr Ala
710 715 720

Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ala Glu Val Pro Ala
725 730 735

Val Glu Glu Asn Val Thr Lys Thr Val Ala Glu Ile Pro Asp Phe
740 745 750

Asn Ala Thr Ala Asp Asp Leu Ser Ala Leu Leu Gln Pro Ser Glu
755 760 765

Val Pro Ala Val Glu Glu Asn Ala Ala Glu Ile Thr Leu Glu Thr
770 775 780

Pro Asp Ser Asn Thr Ser Glu Ala Asp Ala Leu Pro Asp Phe Leu
785 790 795

Lys Asp Gly Glu Glu Glu Thr Val Asp Trp Ser Ile Tyr Leu Ser
800 805 810

Glu Glu Asn Ile Pro Asn Asn Ala Asp Thr Ser Phe Pro Ser Glu
815 820 825

Ser Val Gly Ser Asp Ala Pro Ser Glu Ala Lys Tyr Asp Leu Ala
830 835 840

Glu Met Tyr Leu Glu Ile Gly Asp Arg Asp Ala Ala Ala Glu Thr
845 850 855

Val Gln Lys Leu Leu Glu Glu Ala Glu Gly Asp Val Leu Lys Arg
860 865 870

Ala Gln Ala Leu Ala Gln Glu Leu Gly Ile Stop
875 880

SEQ ID NO 3

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                                        AAAT  GTTCGAATTA  CGAACAAGTT      24
AAATCCGGAG  CTTATCTCGG  GTCTATGGTT  TTAATTCTGT  TTGTCGTTAT      74
TTACGGCTTC  AGGCTGCTGG  TTAATTTCTT  AAAAGACATA  GGCAAAGTAG     124

TTT TGC AGA ACC AGC AAG AAT GAT GAT CGA ATA ATA AAA TTT AGG   169
Phe Cys Arg Thr Ser Lys Asn Asp Asp Arg Ile Ile Lys Phe Arg
1               -5                  -10                 -15

CCA TCT AAA TTA AAG TTT TTT GAA TCT ACA GGA TAT AGA AAA ATC   214
Pro Ser Lys Leu Lys Phe Phe Glu Ser Thr Gly Tyr Arg Lys Ile
                -20                 -25                 -30

AAT AAT GAA TTT TCT AAA TTC ACA GAA GCG GCA AAT GTC GAA CAT   259
Asn Asn Glu Phe Ser Lys Phe Thr Glu Ala Ala Asn Val Glu His
                -35                 -40                 -45

ATC CCC ACG GGC GCA AAA GCC CGA ATC AAC GCA AAG ATA ACC GCC   304
Ile Pro Thr Gly Ala Lys Ala Arg Ile Asn Ala Lys Ile Thr Ala
                -50                 -55                 -60

AGC GTA TCC CGC GCC GCC GTC TTG TCA GGA GTC GGC AAA CTT GCC   349
Ser Val Ser Arg Ala Ala Val Leu Ser Gly Val Gly Lys Leu Ala
                -65                 -70                 -75

CGC TTA GGC GCG AAA TTA AGC ACA AGG GCA GTT CCT TAT GTC GGA   394
Arg Leu Gly Ala Lys Leu Ser Thr Arg Ala Val Pro Tyr Val Gly
                -80                 -85                 -90

ACA GCC CTT TTA GCC CAT GAC GTA TAC GAA ACT TTC AAA GAA GAC   439
Thr Ala Leu Leu Ala His Asp Val Tyr Glu Thr Phe Lys Glu Asp
                -95                 100                 105

ATA CAG GCA CAA GGC TAC CAA TAC GAC CCC GAA ACC GAC AAA TTT   484
Ile Gln Ala Gln Gly Tyr Gln Tyr Asp Pro Glu Thr Asp Lys Phe
                110                 115                 120

GTA AAA GGC TAC GAA TAT AGT AAT TGC CTT TGG TAC GAA GAC AAA   529
Val Lys Gly Tyr Glu Tyr Ser Asn Cys Leu Trp Tyr Glu Asp Lys
                125                 130                 135

AGA CGT ATT AAT AGA ACC TAT GGC TGC TAC GGC GTT GAC AGT TCG   574
Arg Arg Ile Asn Arg Thr Tyr Gly Cys Tyr Gly Val Asp Ser Ser
                140                 145                 150

ATT ATG CGC CTT ATG TCC GAT GAC AGC AGA TTC CCC GAA GTC AAA   619
Ile Met Arg Leu Met Ser Asp Asp Ser Arg Phe Pro Glu Val Lys
                155                 160                 165

GAA TTG ATG GAA AGC CAA ATG TAT AGG CTG GCA CGT CCG TTT TGG   664
Glu Leu Met Glu Ser Gln Met Tyr Arg Leu Ala Arg Pro Phe Trp
                170                 175                 180

AAT TGG CAT AAA GAA GAA CTG AAT AAA TTA AGT TCT TTG GAT TGG   709
Asn Trp His Lys Glu Glu Leu Asn Lys Leu Ser Ser Leu Asp Trp
                185                 190                 195

AAT AAT TTT GTT TTA AAT CGT TGC ACA TTT AAT TGG AAT GGC GGA   754
Asn Asn Phe Val Leu Asn Arg Cys Thr Phe Asn Trp Asn Gly Gly
                200                 205                 210

GAT TGT TTG GTC AAT AAA GGT GAT GAT TTC AGA AAT GGG GCT GAT   799
Asp Cys Leu Val Asn Lys Gly Asp Asp Phe Arg Asn Gly Ala Asp
                215                 220                 225

TTT TCC CTT ATT CGC AAT TCA AAA TAC AAA GAA GAA ATG GAT GCC   844
Phe Ser Leu Ile Arg Asn Ser Lys Tyr Lys Glu Glu Met Asp Ala
                230                 235                 240
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AAA AAG CTG GAA GAG AT[redacted]TA TCG TTG AAA GTC GAT GCC AAT [redacted]C 889
Lys Lys Leu Glu Glu Ile Leu Ser Leu Lys Val Asp Ala Asn Pro
245 250 255

GAC AAA TAC ATA AAG GAA ACC GGT TAT CCC GGT TAT TCC GAA AAA 934
Asp Lys Tyr Ile Lys Glu Thr Gly Tyr Pro Gly Tyr Ser Glu Lys
260 265 270

GTA GAA GTC GCA CCC GGA ACA AAA GTG AAT ATG GGT CCC GTC ACG 979
Val Glu Val Ala Pro Gly Thr Lys Val Asn Met Gly Pro Val Thr
275 280 285

GAC AGG AAC GGG AAT CCC GTT CAG GTT GTC GCA ACA TTC GGC AGG 1024
Asp Arg Asn Gly Asn Pro Val Gln Val Val Ala Thr Phe Gly Arg
290 295 300

GAT TCG CAA GGC AAC ACC ACG GTG GAT GTT CAA GTA ATC CCG CGT 1069
Asp Ser Gln Gly Asn Thr Thr Val Asp Val Gln Val Ile Pro Arg
305 310 315

CCC GAC TTG ACC CCC GGA AGC GCG GAA GCA CCG AAC GCA CAG CCG 1114
Pro Asp Leu Thr Pro Gly Ser Ala Glu Ala Pro Asn Ala Gln Pro
320 325 330

CTG CCC GAA GTA TCG CCC GCC GAA AAC CCC GCA AAC AAC CCG AAC 1159
Leu Pro Glu Val Ser Pro Ala Glu Asn Pro Ala Asn Asn Pro Asn
335 340 345

CCC AAT GAG AAC CCC GGC ACG AGC CCC AAT CCC GAA CCC GAC CCC 1204
Pro Asn Glu Asn Pro Gly Thr Ser Pro Asn Pro Glu Pro Asp Pro
350 355 360

GAT TTG AAT CCC GAT GCA AAT CCC GAT ACG GAC GGA CAG CCC GGC 1249
Asp Leu Asn Pro Asp Ala Asn Pro Asp Thr Asp Gly Gln Pro Gly
365 370 375

ACA AGA CCC GAT TCC CCC GCC GTT CCG GGA CGC ACA AAC GGC AGG 1294
Thr Arg Pro Asp Ser Pro Ala Val Pro Gly Arg Thr Asn Gly Arg
380 385 390

GAC GGC AAA GAC GGA AAG GAC GGC AAA GAT GGC GGC CTT TTG TGC 1339
Asp Gly Lys Asp Gly Lys Asp Gly Lys Asp Gly Gly Leu Leu Cys
395 400 405

AAA TTC TTC CCC GAC ATT CTC GCT TGC GAC AGG CTG CCC GAG TCC 1384
Lys Phe Phe Pro Asp Ile Leu Ala Cys Asp Arg Leu Pro Glu Ser
410 415 420

AAT CCG GCA GAA GAT TTA AAT CTG CCG TCT GAA ACC GTC AAT GTA 1429
Asn Pro Ala Glu Asp Leu Asn Leu Pro Ser Glu Thr Val Asn Val
425 430 435

GAG TTT CAG AAA TCA GGA ATC TTT CAA GAT TCC GCA CAG TGT CCC 1474
Glu Phe Gln Lys Ser Gly Ile Phe Gln Asp Ser Ala Gln Cys Pro
440 445 450

GCA CCT GTC ACT TTC ACA GTG ACT GTG CTT GAT TCA AGC AGG CAG 1519
Ala Pro Val Thr Phe Thr Val Thr Val Leu Asp Ser Ser Arg Gln
455 460 465

TTC GCG TTC AGC TTT GAG AAC GCA TGT ACC ATA GCC GAA CGG CTA 1564
Phe Ala Phe Ser Phe Glu Asn Ala Cys Thr Ile Ala Glu Arg Leu
470 475 480

AGG TAC ATG CTT CTC GCC CTT GCT TGG GCG GTT GCC GCC TTT TTT 1609
Arg Tyr Met Leu Leu Ala Leu Ala Trp Ala Val Ala Ala Phe Phe
485 490 495

TGT ATC CGC ACA GTA TCT CGT GAA GTC TAG 1639
Cys Ile Arg Thr Val Ser Arg Glu Val Stop
500 504

SEQIDNO 4

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Phe Cys Arg Thr Ser Lys Asn Asp Asp Arg Ile Ile Lys Phe Arg
1               -5                  -10                 -15

Pro Ser Lys Leu Lys Phe Phe Glu Ser Thr Gly Tyr Arg Lys Ile
                -20                 -25                 -30

Asn Asn Glu Phe Ser Lys Phe Thr Glu Ala Ala Asn Val Glu His
                -35                 -40                 -45

Ile Pro Thr Gly Ala Lys Ala Arg Ile Asn Ala Lys Ile Thr Ala
                -50                 -55                 -60

Ser Val Ser Arg Ala Ala Val Leu Ser Gly Val Gly Lys Leu Ala
                -65                 -70                 -75

Arg Leu Gly Ala Lys Leu Ser Thr Arg Ala Val Pro Tyr Val Gly
                -80                 -85                 -90

Thr Ala Leu Leu Ala His Asp Val Tyr Glu Thr Phe Lys Glu Asp
                -95                 100                 105

Ile Gln Ala Gln Gly Tyr Gln Tyr Asp Pro Glu Thr Asp Lys Phe
                110                 115                 120

Val Lys Gly Tyr Glu Tyr Ser Asn Cys Leu Trp Tyr Glu Asp Lys
                125                 130                 135

Arg Arg Ile Asn Arg Thr Tyr Gly Cys Tyr Gly Val Asp Ser Ser
                140                 145                 150

Ile Met Arg Leu Met Ser Asp Asp Ser Arg Phe Pro Glu Val Lys
                155                 160                 165

Glu Leu Met Glu Ser Gln Met Tyr Arg Leu Ala Arg Pro Phe Trp
                170                 175                 180

Asn Trp His Lys Glu Glu Leu Asn Lys Leu Ser Ser Leu Asp Trp
                185                 190                 195

Asn Asn Phe Val Leu Asn Arg Cys Thr Phe Asn Trp Asn Gly Gly
                200                 205                 210

Asp Cys Leu Val Asn Lys Gly Asp Asp Phe Arg Asn Gly Ala Asp
                215                 220                 225

Phe Ser Leu Ile Arg Asn Ser Lys Tyr Lys Glu Glu Met Asp Ala
                230                 235                 240

Lys Lys Leu Glu Glu Ile Leu Ser Leu Lys Val Asp Ala Asn Pro
                245                 250                 255

Asp Lys Tyr Ile Lys Glu Thr Gly Tyr Pro Gly Tyr Ser Glu Lys
                260                 265                 270

Val Glu Val Ala Pro Gly Thr Lys Val Asn Met Gly Pro Val Thr
                275                 280                 295

Asp Arg Asn Gly Asn Pro Val Gln Val Val Ala Thr Phe Gly Arg
                290                 295                 300

Asp Ser Gln Gly Asn Thr Thr Val Asp Val Gln Val Ile Pro Arg
                305                 310                 315

Pro Asp Leu Thr Pro Gly Ser Ala Glu Ala Pro Asn Ala Gln Pro
                320                 325                 330

Leu Pro Glu Val Ser Pro Ala Glu Asn Pro Ala Asn Asn Pro Asn
                335                 340                 345
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Pro Asn Glu Asn Pro Gl[redacted]hr Ser Pro Asn Pro Glu Pro Asp[redacted]ro
350 355 360

Asp Leu Asn Pro Asp Ala Asn Pro Asp Thr Asp Gly Gln Pro Gly
365 370 375

Thr Arg Pro Asp Ser Pro Ala Val Pro Gly Arg Thr Asn Gly Arg
380 385 390

Asp Gly Lys Asp Gly Lys Asp Gly Lys Asp Gly Gly Leu Leu Cys
395 400 405

Lys Phe Phe Pro Asp Ile Leu Ala Cys Asp Arg Leu Pro Glu Ser
410 415 420

Asn Pro Ala Glu Asp Leu Asn Leu Pro Ser Glu Thr Val Asn Val
425 430 435

Glu Phe Gln Lys Ser Gly Ile Phe Gln Asp Ser Ala Gln Cys Pro
440 445 450

Ala Pro Val Thr Phe Thr Val Thr Val Leu Asp Ser Ser Arg Gln
455 460 465

Phe Ala Phe Ser Phe Glu Asn Ala Cys Thr Ile Ala Glu Arg Leu
470 475 480

Arg Tyr Met Leu Leu Ala Leu Ala Trp Ala Val Ala Ala Phe Phe
485 490 495

Cys Ile Arg Thr Val Ser Arg Glu Val Stop
500 504

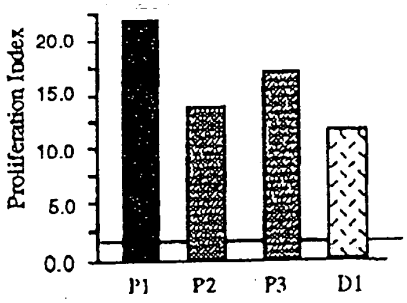

Fig 1

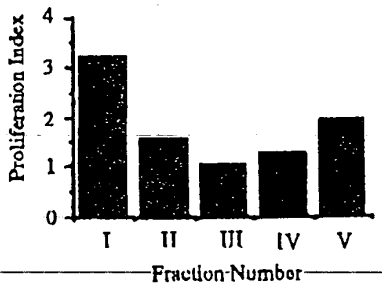

Fig 2

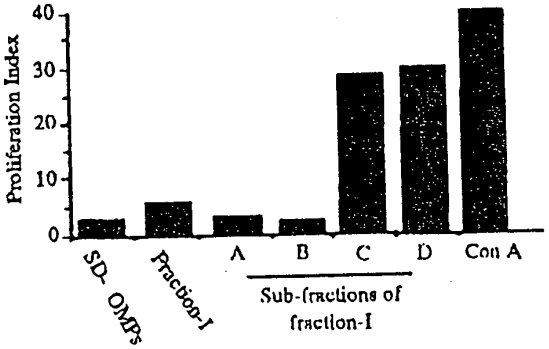

Fig 3

PCT NO : GB99/02205

Form 23/77 : 9/7/99

AGENT : Swindell & Pearson